IFN-γ Induces Apoptosis in Ovarian Cancer Cells in Vivo and in Vitro

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ABSTRACT

Purpose: The purpose of this study was to compare in vitro and in vivo responses of primary human tumor cells to IFN-γ.

Experimental Design: IFN-γ may have therapeutic activity in patients with ovarian cancer. We showed previously that this cytokine had direct antiproliferative activity against human ovarian cancer cell lines and xenografts in nude mice. To further understand the role of IFN-γ in ovarian cancer, we compared its action on 8 ovarian cancer cell lines with the response of 14 primary cultures of ovarian tumor cells isolated from patients with ascitic disease. A pilot clinical study was then conducted to see whether IFN-γ would also induce apoptosis in human tumor cells in vivo. Six patients with ascites and advanced disease were given IFN-γ by i.p. injection, and sequential samples of ascites were analyzed.

Results: IFN-γ had antiproliferative activity in 8 of 8 ovarian cancer cell lines and 11 of 14 primary cultures. This activity was dose related, and cleaved poly(ADP-ribose) polymerase in protein isolates provided evidence of apoptosis. In the clinical study, there was a 3 log_{10} pharmacokinetic advantage in peritoneal compared with plasma levels of IFN-γ. In two of six patients, there was a 90% reduction in tumor cells in ascites after IFN-γ treatment, and this was related to clinical benefit as assessed by intervals between paracentesis. In all six patients, there were increased amounts of cleaved poly(ADP-ribose) polymerase in protein extracts of ascitic cells sampled during IFN-γ treatment.

Conclusions: IFN-γ induces apoptosis in vitro and in vivo in human epithelial ovarian cancer.

INTRODUCTION

IFN-γ is a potent immunomodulatory, antiviral, and antiproliferative cytokine that has anticancer activity. IFN-γ directly inhibits human tumor cell growth and induces apoptosis. Extensive experiments in a range of animal cancer models suggest that endogenous IFN-γ may be involved in immune surveillance of tumors via a combination of lymphocyte-mediated responses, direct actions on tumor cells, and inhibition of tumor angiogenesis (reviewed in Ref. 1). We demonstrated previously that human IFN-γ can directly inhibit the growth of human ovarian tumor cells in vitro and human ovarian cancer xenografts growing in nude mice. IFN-γ had antiproliferative activity in three of four ovarian cancer cell lines, and this was associated with the induction of apoptosis (2). The effects were not immediate but required 2–3 days of exposure to IFN-γ for an irreversible effect on cell survival. Treatment of two of three human ovarian cancer xenografts with IFN-γ at clinically relevant doses induced tumor cell growth arrest and apoptosis. Likewise, this effect was not immediate but required 14–21 days of continuous treatment to significantly increase mouse survival (3).

IFN-γ has been used in the treatment of patients with advanced ovarian cancer. In a Phase II trial, 108 patients with residual disease documented at second-look laparotomy after first-line cisplatin-based chemotherapy were treated with i.p. IFN-γ twice a week for 3–4 months (4). Ninety-eight patients were assessable, with 23 patients achieving a complete response, and 8 patients achieving a partial response. Age and tumor size were the only factors affecting response to IFN-γ. The probability of IFN-γ response was independent of previous response to first-line chemotherapy. In a randomized Phase III study, 148 women treated with cisplatin and cyclophosphamide as first-line chemotherapy for ovarian cancer were randomly allocated to receive additional IFN-γ three times weekly on alternate weeks (5). IFN-γ administration was associated with a significant increase in progression-free survival, but an observed increase in overall survival was not statistically significant. Response to IFN-γ was not related to volume of disease.

The mechanisms of action of IFN-γ treatment in patients with ovarian cancer are not known. Because we had evidence from ovarian cancer cell lines and xenografts that direct action of IFN-γ on tumor cells might be important, the aim of the work described in this paper was to investigate the direct actions of IFN-γ on primary ovarian epithelial cancer cells, both in vitro and in vivo in patients with advanced ascitic disease.

MATERIALS AND METHODS

Ovarian Cancer Cell Lines. The OAW42 cell line was derived from the ascites of a patient with serous adenocarcinoma treated previously with cisplatin (Ref. 6; obtained from...
the European Tissue Culture Collection, Porton Down, United Kingdom). Ovcar-3 originated from the ascites of a patient with poorly differentiated papillary adenocarcinoma treated previously with chemotherapy (7). Ovcar-4 originated from the ascites of a patient with adenocarcinoma treated previously with chemotherapy (8). Ovcar-5 was derived from the ascites of a patient with untreated adenocarcinoma (9). The Ovcar cell lines were obtained from T. Hamilton (Fox Chase Cancer Institute, PA). PEO1 was derived from ascites in a patient with poorly differentiated adenocarcinoma. The PEO1 (CDDP) cell line was obtained by continuous exposure of the PEO1 cell line to increasing concentrations of cisplatin. PEO14 was derived from the ascites of a patient with a well-differentiated serous adenocarcinoma who had not received prior drug therapy. PEO16 originated from the ascites of a patient with serous adenocarcinoma who had been treated previously with radiotherapy. All of the PEO cell lines were raised in the Cancer Research UK Laboratories in Edinburgh (10). SW626 is an adenocarcinoma cell line initially described in Ref. 11. This cell line was obtained from the American Type Culture Collection (Manassas, VA).

**rhIFN-γ.** rhIFN-γ for the in vitro experiments was provided by Roussel UCLAF as a lyophilized preparation (20 × 10⁶ units/vial) and was >95% pure. It was diluted in water and stored in aliquots at −70°C until use. Endotoxin levels were less than 0.24 endotoxin units/vial, and the specific activity was 2 × 10⁷ units/mg protein. rhIFN-γ 1b (200 μg/ml; specific activity, 2 × 10⁷ units/mg protein) was used for the clinical trial (Boehring Ringer Ingelheim, Bracknell, United Kingdom). It was supplied as an isotonic solution, stored at −4°C until use and diluted to 50 ml in normal saline immediately before administration. The IFN-γ was administered by slow i.p. injection on the opposite side of the abdomen to the ascitic drain at a site that had been marked by ultrasound.

**Ascites Sample Collection and Preparation.** Samples were collected from patients with advanced ovarian cancer at the time of therapeutic paracentesis or surgery. Written consent was obtained from all patients. Cells were collected by centrifugation at 500 × g for 5 min. If necessary, red cells were removed by centrifugation through Ficoll according to the manufacturer’s instructions (Sigma). Cells were resuspended in 20% FCS with 10% DMSO and stored in liquid nitrogen.

Malignant cells were purified from the ascitic cell suspension according to the method of Hurteau et al. (12). In brief, cells were layered onto discontinuous density gradients of Percoll (Pharmacia, Uppsala, Sweden) and centrifuged at 850 × g, and the top layer of cells was harvested. Cells were then mixed with CD45-coated Dynabeads (Dynal Ltd.) at 4°C for 30 min. The beads were magnetically removed. Cytospins were performed on the purified samples to assess composition. Samples were discarded unless >90% of cells stained with epithelial markers (cytokeratin antibodies used were AUA1, BerEP4, and EMA).

**Cytospins.** A small aliquot of cells was removed before freezing and passed 5–10 times through a 19-gauge needle to break up clumped cells. Cells were suspended at a concentration of 5–7 × 10⁵ cells/ml. One hundred μl of the cell suspension were spun onto electrostatically charged slides (“Superfrost,” BDH Laboratories) at 500 rpm for 5 min using a cytocentrifuge (Shandon, Runcorn, United Kingdom). Slides were air dried, fixed in formal saline, rinsed in distilled water, and stored at room temperature. Alternatively, the cytospins used for cleaved PARP were fixed in 4% paraformaldehyde (Sigma), and the immunohistochemistry was performed at once.

**Tissue Culture.** Cell lines were grown in a humidified atmosphere in 5% CO₂ at 37°C under pyrogen-free conditions. Pipettes were plastic and individually wrapped. Tissue culture media and fetal calf serum (Life Technologies, Inc.) were chosen for their low endotoxin content, and plastic tubes and flasks were used at all times. Cell lines were grown in RPMI 1640 with glutamine (Life Technologies, Inc.), FCS (10%), penicillin (100 units/ml), and streptomycin (100 μg/ml; Sigma). Primary cells were cultured in RPMI 1640 with glutamine FCS (20%), penicillin (100 units/ml), streptomycin (100 μg/ml), and amphotericin B (0.625 μg/ml; Sigma).

Primary cells were seeded at 2 × 10⁶ cells/ml into “Primaria”-coated tissue culture dishes (Marathon Laboratory Supplies, London, United Kingdom). After 24–48 h of culture, nonadherent cells and tissue culture medium were aspirated, and adherent cells were washed in RPMI 1640 and treated with IFN-γ. At the time that the experiment was harvested, cells were scraped from an untreated well of the dish, and cytospins were prepared.

**Crystal Violet Proliferation Assay.** Cell lines were seeded at 5 × 10⁵ cells/well into 96-well tissue culture dishes. Primary cells were seeded at 5 × 10⁴ cells/well. Six to 12 wells were treated per concentration time point. Cells were allowed to settle overnight in the dishes and then treated with IFN-γ-containing medium. After 8 days, the medium was discarded, and adherent cells were washed in PBS and fixed in methanol. A 1% aqueous solution of crystal violet was added for 20 min. Cells were washed again, and then the remainder of the dye was resolubilized in methanol. The transmission of each well was read at 600 nm (Bio-Rad 2550 EIA Reader).

**Protein Extraction.** Proteins were extracted according to the method supplied by Enzyme System Products (Enzyme System Products, Livermore, CA). Briefly, adherent cells were scraped from one well of a 6-well plate, and adherent and floating cells were collected by centrifugation. A small aliquot of cells was removed for protein estimation, which was performed using the Bio-Rad protein assay system (Bio-Rad Laboratories, Munich, Germany) according to the manufacturer’s instructions. The remainder of the cells were suspended in loading buffer [62.5 mM Tris (pH 6.8), 6 mM urea, 10% glycerol, 2% SDS, 0.003% bromphenol blue, and 5% 2-mercaptoethanol freshly added]. Cells were sonicated on ice and stored at −40°C until analysis.

**PAGE.** Electrophoresis was performed using standard conditions as described previously (13). Ten percent gels were used, and 25 μg of protein were loaded per sample. Protein lysates were run at 110 V for 2 h using 1× running buffer (25 mM Tris, 192 mM glycine, and 0.1% SDS). Full-range Rainbow

4 The abbreviations used are: PARP, poly(ADP-ribose) polymerase; rhIFN, recombinant human IFN; FAM, carboxyfluorescein; FMK, fluoromethyl ketone; NK, natural killer.
Markers (Amersham International) were used to determine product size.

**Western Blotting.** Protein was transferred to nitrocellulose (Hybond-ECL; Amersham International) pre-equilibrated in transfer buffer (25 mm Tris, 192 mm glycine, and 20% methanol) according to a previously described method (14). Blots were incubated using a Trans-Blot electrophoretic transfer cell (Bio-Rad) for 2 h at 55°V at 4°C. Blots were incubated in blocking reagent, 5% nonfat milk powder and 0.1% Tween 20 in PBS, and then washed. Antibodies were diluted in 3% nonfat milk powder and 0.1% Tween 20 in PBS. Blots were exposed to the primary antibody at 4°C with agitation overnight and to the secondary antibody at room temperature with agitation for 1 h. The Amersham ECL system was used for detection.

**Antibodies.** All antibodies were purchased from Dako Ltd., unless otherwise indicated. Anti-fibroblast antibody was used at a 1:20 dilution, CD15 (primarily granulocytes) was used at a 1:25 dilution, CD45 (primarily leukocytes) was used at a 1:200 dilution, and CD68 (macrophages) was used at a 1:50 dilution. Three antibodies were used to stain epithelial cells: (a) AU1 undiluted (made by Cancer Research UK); (b) BerEP4; and (c) EMA. Appropriate second layers were used at dilutions as recommended by the manufacturers. PARP-1 (Enzyme System Products, Livermore, CA); was used at 1:10,000 dilution. Antibody to cleaved PARP (Cell Signaling Technology, Beverly, MA) was used at a 1:1000 dilution. β-Actin was obtained from Sigma at 1:1000.

**Fluorescence-activated Cell-sorting Analysis.** APO LOGIX FAM caspase detection kits (Cell Technology, MN) were used to detect active caspase 8 (FAM-LETD-FMK) and active caspase 9 (FAM-LEHD-FMK) in situ, according to the manufacturer’s instructions (488 nm excitation; green fluorescence emission). Briefly, 10 μl of 30× Working Dilution FAM-Peptide-FMK was added per 300 μl of medium and incubated, protected from light, for 1 h at 37°C under 5% CO2. Adherent cells were washed twice with 1× Working Dilution Wash Buffer, harvested by trypsinization, and collected by centrifugation. Nonadherent cells were collected by centrifugation and washed twice. Both adherent and nonadherent cells were resuspended together in 400 μl of 1× Working Dilution Wash Buffer. TO-PRO-3 (633 nm excitation; red fluorescence emission) was used to discriminate dead cells. Cells were analyzed on a FACScalibur flow cytometer using CellQuest Software (Becton Dickinson).

**Clinical Study.** The clinical study was reviewed by the Western General Hospital Ethics Committee, and all patients gave written, informed consent. Patients with ovarian cancer admitted to hospital for therapeutic paracentesis were eligible to enter the study. At the time of insertion of the drain for paracentesis, IFN-γ (20 × 10^6 units/m², calculated using the patient’s weight before the development of ascites) was inserted into the abdomen by abdominal puncture at the opposite side of the abdomen to the ascitic drain. Ascites was drained over the following 3–4 days, and aliquots of the ascites and of venous blood were taken for analysis. A second dose of IFN-γ was inserted at the time of drain removal. Clinical details of the six patients in the pilot trial are given in Table 1.

**IFN-γ Assay.** Concentrations of IFN-γ in patients’ plasma and ascitic fluid were determined using a Quantikine human IFN-γ assay kit (R&D Systems, Minneapolis, MN) according to manufacturer’s instructions.

**Immunocytochemistry.** Conventional methods were used. Briefly, slides were pretreated with trypsin or microwaved in boiling citrate buffer. Slides were incubated for 2 h in the primary antibody and then incubated for 1 h each in the biotinylated secondary antibody and streptavidin-peroxidase. Slides were briefly exposed to 3,3'-diaminobenzidine tetrachloride in PBS with 0.06% hydrogen peroxide (H2O2). Counterstaining was performed with hematoxylin. Slides were washed, dehydrated, and mounted and then examined through a ×40 objective with a ×12.5 eyepiece. The number of nucleated cells staining in each of 5–10 high-powered fields was counted, and the mean ± SE of cell populations from each sample was calculated. Immunohistochemistry for cleaved PARP was performed following the manufacturer’s instructions.

### Table 1 Clinical history of patients entered into the pilot study of i.p. IFN-γ

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<th>Patient no.</th>
<th>Stage</th>
<th>Surgery</th>
<th>Previous exposure to chemotherapy</th>
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<td>Yes</td>
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</tr>
<tr>
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<td>IV</td>
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<td>IV</td>
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### RESULTS

**IFN-γ Inhibits the Growth of Ovarian Cancer Cell Lines.** The effect of IFN-γ on the growth of eight ovarian cancer cell lines was assessed. Cells were cultured with 5000 units/ml IFN-γ, and results were expressed as a fraction of control. Fig. 1a shows results obtained after 7–8 days of culture. Significant (P < 0.001) growth inhibition was observed in all of the cell lines. For the majority of cell lines, the full magnitude of growth inhibition was apparent by 4 days of IFN-γ exposure. In the PEO16 cell line, which had a relatively long doubling time, there was no antiproliferative effect at 4 days, and the maximal effect was observed after 8 days of IFN-γ exposure.

The effects of IFN-γ were dose related. A representative curve is shown in Fig. 2a. The majority of cell lines demonstrated a sigmoidal dose–response relationship, and the maximal antiproliferative response was achieved at doses below the highest dose tested. The position of the lower shoulder on the
dose-response curve, at which maximal antiproliferative response was seen, was as low as 10 units/ml IFN-γ/H9253 for Ovcar-4 cells, whereas it was 5000 units/ml for the SW626 line. The median dose at which the maximal antiproliferative response was seen was 200 units/ml.

**IFN-γ Inhibits Growth of Primary Isolates of Ovarian Cancer Cells.** Tumor cells were isolated and cultured from 14 ascitic samples from 13 patients. Fibroblasts were isolated from the same samples from two of these patients but were not significantly growth inhibited by IFN-γ (data not shown).

Growth was determined after exposure of primary cultures to IFN-γ at concentrations of 200 and 5000 units/ml. Fig. 1b shows growth of cells after 7 or 8 days of exposure to 5000 units/ml IFN-γ (patients were labeled A–Ma). IFN-γ at 200 units/ml had a significant antiproliferative effect in 11 of 14 cultures (79%), whereas at 5000 units/ml, a significant antiproliferative effect was seen in 10 of the 14 cultures (71%). The median cell number at these concentrations was 47% and 42% of controls, respectively. The effects of IFN-γ were again dose related. A representative example of a dose-response curve is shown in Fig. 2b.

**IFN-γ Induces Apoptosis in Cell Lines and in Primary Tumor Cell Isolates.** PARP is involved in DNA repair. It is targeted for proteolytic cleavage by the caspases during apoptosis, marking an irreversible commitment to cell death. After exposure of Ovcar-4 ovarian cancer cell line (a) or a primary culture from patient 1 (b) to IFN-γ at doses ranging from 1 to 10,000 units/ml for 4 days. Ovcar-4 cells were seeded at 5 × 10^4 cells/well, and primary cells from patient 9 were seeded at 5 × 10^4 cells/well into 96-well tissue culture dishes. Six to 12 wells were treated per concentration time point. The transmission of each well was read at 600 nm.

Fig. 1 The antiproliferative response of ovarian cancer cell lines and primary cultures of ovarian cancer cells to IFN-γ. a, cells from eight ovarian cancer cell lines. b, cells from 14 primary cultures were obtained from patients A–Ma (cells from patient M were obtained on two different occasions, M and Ma). All cells were exposed to IFN-γ (5000 units/ml) for 7–8 days. Growth is expressed as a fraction of control values. Cell lines were seeded at 5 × 10^5 cells/well, and primary cells were seeded at 5 × 10^4 cells/well into 96-well tissue culture dishes. Six to 12 wells were treated per concentration time point. After 7–8 days, the medium was discarded, and adherent cells were washed and fixed in methanol before staining with an aqueous solution of crystal violet. The transmission of each well was read at 600 nm.

Fig. 2 The response of primary ovarian tumor cells and ovarian cell lines to a range of doses of IFN-γ. Dose-response relationship after the exposure of Ovcar-4 ovarian cancer cell line (a) or a primary culture from patient 1 (b) to IFN-γ at doses ranging from 1 to 10,000 units/ml for 4 days. Ovcar-4 cells were seeded at 5 × 10^4 cells/well, and primary cells from patient 9 were seeded at 5 × 10^4 cells/well into 96-well tissue culture dishes. Six to 12 wells were treated per concentration time point.
to IFN-γ by fluorescence-activated cell-sorting analysis. Preliminary experiments show both active caspase 8 and 9 were detected in cells from the ovarian cancer cell line PEO1 after 72 h of exposure to IFN-γ (Fig. 3, b and c).

There was also evidence that IFN-γ induced apoptosis in primary tumor cells. Three of the primary tumor cell isolates were exposed to IFN-γ (5000 units/ml) for 96 h, and total cellular protein from all adherent and floating cells was harvested. In two primary cell cultures that were growth inhibited by IFN-γ (patients I and Ma), there was an increase in the proportion of PARP in the cleaved form after IFN-γ exposure, but no effect was seen in an insensitive culture (patient H). This is illustrated in Fig. 4.

The Pharmacokinetics of IFN-γ Administered into the Peritoneum. IFN-γ (20 × 10^6 units/m^2) was given i.p. to six patients with platinum-resistant disease. The pharmacokinetics are shown in Fig. 5. Local administration of IFN-γ was associated with high concentration of IFN-γ within the peritoneum and a 3 log_{10} pharmacokinetic advantage in comparison with the plasma. In all patients, the peak IFN-γ concentration was achieved at 4 h. There was considerable variability (10-fold) within the peaks achieved in the different patients. All patients achieved a peak IFN-γ concentration greater than 1000 units/ml, but this was not maintained for 72 h in any of the patients. Two of the patients (patients 5 and 6) maintained concentrations above 200 units/ml for 72 h.

Cellular Composition of Ascites in Patients Receiving i.p. rhIFN-γ. The cellular composition of the ascites was analyzed at regular time intervals during the ascites collection as shown in Fig. 6. Two of the patient samples (patients 2 and 4) showed little or no tumor cells upon analysis of the cellular composition. Of the four patients with significant numbers of tumor cells, two (patients 1 and 6) showed a reduction in the proportion of malignant cells in the ascites from 72 and 96 h, respectively, after the IFN-γ infusion. In patient 1, the proportion of tumor cells comprised approximately 93% of the total cells in ascites up to 24 h after IFN-γ treatment, but this declined to 2% by 72 h. In patient 6, there was also a reduction in the proportion of tumor cells; 25% of the total cells were tumor cells before treatment, and this percentage was reduced to 2% by 96 h.

IFN-γ Treatment Induces Cleaved PARP in Ascites Cells. Protein lysates from cells recovered from ascites during the treatment time course were analyzed by Western blotting for cleaved PARP. The results are shown in Fig. 7. All samples showed evidence of PARP cleavage, and this generally in-
creased over the treatment period. In one patient (patient 3), we were also able to study sequential cytospins for cleaved PARP by immunohistochemistry. At the start of treatment, 4.4% cells stained for cleaved PARP. This increased to 9.3% 24 h after treatment and 15.2% 48 h after treatment. The percentage of cleaved PARP-positive cells was 9.5% by 72 h. A typical field is shown in Fig. 8 (baseline and 72 h after treatment commenced).

The Clinical Consequences of i.p. IFN-γ. All six of the patients entered into the clinical study had cisplatin- or carboplatin-refractory disease. Five of the patients also had paclitaxel-refractory disease (see Table 1). Two of the six patients experienced grade 3 toxicity in relation to the IFN-γ administration. This consisted of malaise and abdominal pain (one patient) and constipation (one patient). Therefore, 20 × 10⁶ units/m² was the maximal tolerated dose in this group of patients. Despite the advanced stage of disease at the time of IFN-γ administration, patients 1 and 6 appeared to achieve clinical disease stabilization after the treatment, as detailed below. These two patients also exhibited a reduction in tumor cells in the peritoneum as outlined earlier.

Patient 1, who had recurrent ovarian cancer, required therapeutic paracentesis on three occasions over a 4-month period, during which period she received no disease-modifying therapies. On the third occasion she also received IFN-γ. After the IFN-γ, she experienced acute, severe abdominal pain and a high fever. Hence only one dose of IFN-γ was administered. It was 4 months until she again required paracentesis, and over this period she received no disease-modifying therapies. Five months after the IFN-γ she was commenced on hormonal treatment for her disease. She died 7 months after entry into the study.

Patient 2 was treated with IFN-γ when she developed symptomatic ascites 2 months after completing salvage therapy for recurrent ovarian cancer. She reaccumulation of the ascites 3 weeks later and thus was recommenced on chemotherapy. She died 11 months after study entry.

Patient 3 was treated with IFN-γ when she developed ascites while on chemotherapy for persistent ovarian cancer. Ascites reaccumulated, and she died 2 months after study entry, having received no other anticancer therapies.

Patient 4 developed symptomatic ascites shortly after the end of a course of palliative chemotherapy for recurrent disease. She was treated with IFN-γ when she had a second therapeutic paracentesis within a month. Two weeks later, she was admitted with septicemia and died.

Patient 5 developed symptomatic ascites 4 months after completion of adjuvant chemotherapy for her disease. She was treated with IFN-γ at the time of paracentesis. She was readmitted 3 weeks later with subacute bowel obstruction. This was not amenable to surgical intervention, and she died of her disease 3 months after entry into the study.

Patient 6 was on palliative chemotherapy (liposomal doxorubicin) for recurrent ovarian cancer. She required monthly therapeutic paracentesis over a 7-month period before and during her chemotherapy. On the seventh occasion she also received i.p. IFN-γ. She only received one dose of treatment due to the development of fever and myalgia. It was 3.5 months until she required further paracentesis, and over this period she received no other anticancer therapies. She was then commenced on oral etoposide. She was unable to tolerate this due to myelosuppression and was commenced on a hormonal agent. She died 9 months after entry into the study.

In a nonrandomized trial of so few patients it is inappropriate to draw conclusions about clinical benefit, but we were impressed that for patients 1 and 6, there was a significant increase in the time before subsequent paracentesis was required. We attribute this to IFN-γ administration. Before receiving IFN-γ, both of these patients had required approximately monthly drainage, and after IFN-γ, this interval was extended to
4 and 3.5 months, respectively. Both patients were grateful for the avoidance of hospital admission at such a critical time in their illness. Patients 4 and 5 were not assessable due to rapid progression of their disease. Patients 2 and 3 did not appear to have clinical benefit from the treatment.

**DISCUSSION**

We have previously shown that IFN-γ has direct antiproliferative activity in some ovarian cancer cell lines and in human ovarian cancer xenografts grown in nude mice (2). We have now confirmed these results in a larger panel of ovarian cancer cell lines and in primary tumor cells isolated from ascites. IFN-γ had an antiproliferative effect in 11 of 14 primary tumor cell cultures, and the dose-response curve was similar to that of cell lines.

Ascites has been used extensively as a source of cells for primary culture of human ovarian cancer. Tumor cells within ascites are phenotypically very similar to those in solid tumors (15), although they do show some discordance in the expression of p53 (16) and down-regulation of E-cadherin expression (17).

We used assays for cleaved PARP as an indication of which samples responded to IFN-γ with an increase in apoptosis. The proportion of cleaved PARP increased over time in *in vitro* experiments after exposure to IFN-γ, both in sensitive cell lines and primary cells. Moreover, in another IFN-γ-sensitive cell line (PE01), we found evidence of both caspase 8 and caspase 9 activation at 72 h. It is not clear from the literature which caspase pathway is dominant after treatment of cells with conventional chemotherapeutic agents. Whereas several reports suggest that caspase 8 activation is critical in drug-induced apoptosis in ovarian cancer, others suggest that caspase 9 activation is the dominant pathway in testicular cancer (e.g., Ref. 18). Novel therapies that are able to target both apoptotic pathways may be of benefit to patients who prove to be resistant to conventional chemotherapy agents. Experiments are under way to establish whether IFN-γ initiates apoptosis in ovarian cancer cells using a death receptor-initiated pathway.

We went on to conduct a pilot clinical study in six patients. The purpose of this Phase I study was to compare *in vitro* and *in vivo* responses of primary human tumor cells to IFN-γ. By adopting the same dose as had been used in a previous clinical trial of i.p. IFN-γ, we also wished to investigate whether concentrations achieved *in vivo* would be sufficient to maximize the direct action of the IFN-γ on tumor cells.

IFN-γ was administered at the time of paracentesis into the opposite side of the abdomen, and the ascites was slowly drained over the following 3–4 days. All patient samples showed evidence of PARP cleavage, irrespective of clinical response. The timing of induction of PARP cleavage in the two patients who achieved clinical benefit from the IFN-γ treatment was similar to the kinetics of the appearance of cleaved PARP seen in the primary cells and the cell lines treated *in vitro* with IFN-γ. Because we did not have enough material to separate the tumor cells from other cells in ascites, we cannot be sure that apoptosis was just occurring in the tumor cells. However, using immunohistochemistry, we could clearly detect PARP staining in cells with the morphological appearance of tumor cells.

Tumor response was not formally measured in this clinical trial. All patients had CA125 measurements taken at baseline, during paracentesis, and at follow up. CA125 is closely related to tumor response in patients undergoing chemotherapy (19). This has not been demonstrated after treatment with biological therapies. Because a number of cytokines, including IFN-γ, have been shown to directly stimulate CA125 production from malignant cells (20), CA125 monitoring may not be such a useful surrogate marker in the biological therapies. Peritoneal trauma, such as paracentesis, can also stimulate CA125 production (21). CA125 responses in our patients included a marked rise after paracentesis, stabilization, and a marked fall. They appeared unrelated to the clinical course of the patients and hence have not been presented here. We have therefore inferred clinical benefit from the subsequent clinical course of patients while they remained off other anticancer therapies. It is encouraging to have seen clinical benefit in two patients with otherwise advanced, chemotherapy-resistant disease. Of the patients from
whom the primary tumor cultures were obtained, most of them (82%) were clinically resistant to platinum compounds, and 36% of them were clinically resistant to paclitaxel. The activity of IFN-γ in such disease is encouraging because it suggests that response and resistance to IFN-γ are mediated by different mechanisms to chemotherapy.

In the last few years, a series of experiments have been conducted in IFN-γ gene- and IFN-γ receptor gene-deleted mice bearing a range of transplantable, genetic, and inducible cancers. These experiments provided evidence of a major role for endogenously produced IFN-γ in preventing development of tumors (reviewed in Ref. 1). It is likely that, in the intact host, the actions of IFN-γ involve a combination of immunological responses, direct actions on tumor cells, and inhibition of angiogenesis. On the basis of the animal experiments, Ikeda et al. (1) propose that T cells and NK cells involved in innate immunity may recognize developing tumors and initially produce IFN-γ. This local production of IFN-γ induces angiostatic chemokines

Fig. 6 The change in cellular composition of ascites after i.p. IFN-γ in patients undergoing therapeutic paracentesis for advanced ovarian cancer. Immunohistochemistry was performed on cytospins prepared from fresh ascites at the time points indicated. Anti-fib (green) = fibroblasts; CD68 (yellow) = macrophages; CD45 (purple) = lymphocytes; CD15 (red) = granulocytes; AUA1 (blue) = epithelial (tumor) cells.
such as IP-10 that could also attract immune effector cells to the tumor site. Newly arrived effector cells will also be induced to produce IFN-γ that may activate cytotoxic functions of NK cells and macrophages and increase production of other antitumor cytokines such as interleukin 12 and interleukin 18. Dead tumor cells may charge the antigen-presenting machinery of the adaptive immune response. Thus, an IFN-γ-rich milieu promotes the development of a CD4+ Th1 antitumor response that can generate a cytotoxic CD8+ T-cell response, helped by the ability of IFN-γ to increase MHC expression on the tumor cells. Locally produced IFN-γ can now have direct antiproliferative and apoptotic actions on the tumor cells. The importance of direct actions of IFN-γ on tumor cells is highlighted by the observation that tumor cells unable to respond to IFN-γ have increased tumorigenicity (1).

How do these experimental data relate to the results reported here in a human epithelial cancer? It is of interest that in a study of the cytokine microenvironment of human epithelial ovarian cancer we never detected expression of IFN-γ, although all tumors and tumor cell lines expressed the IFN-γ receptor (22). Our data show that the IFN-γ receptor complex is functional in ovarian cancer cells. Moreover, our previous experiments using human tumor xenografts growing in nude mice prove that a direct action of IFN-γ can be important because the human IFN-γ used in these experiments is strictly species specific and had no action on the murine host (3).

We therefore suggest that in advanced human ovarian cancer, the tumor cells have evolved so that they no longer induce a host IFN-γ response. However, a majority of these tumor cells can still respond to the antitumor actions of IFN-γ if it is added exogenously. We have proved that the direct actions of IFN-γ are sufficient to induce apoptosis and reduce growth in vitro in a majority of primary tumor cells and established tumor cell lines. When similar concentrations were achieved within the tumor microenvironment, there was a rapid reduction in the number of tumor cells in the ascitic fluid in two of six patients and evidence of peritoneal cell apoptosis in all patients. Ascitic fluid from ovarian cancer patients contains a variable number of tumor cells and CD4+ and CD8+ T cells and macrophages, as well as smaller numbers of NK cells (17). It is possible that in vivo IFN-γ also stimulated T cells and macrophages to mount an antitumor response, and this may be why we saw evidence of apoptosis, even though exposure to IFN-γ was not sustained at levels that are necessary to induce apoptosis in vitro.

We conclude that IFN-γ might be a useful biological treatment of human epithelial ovarian cancer if sustained levels

\[ \text{Fig. 7 Cleaved PARP protein in ascitic cell lysates from patients 1–6 after i.p. IFN-γ administration. Twenty-five μg of protein were loaded per sample, and Western blotting was performed as outlined in “Materials and Methods.” The blots were stripped and reprobed with β-actin as a loading control. Integrated density relative to β-actin is shown as a bar graph below each blot.} \]
of this cytokine could be achieved within the peritoneum by improved protein or gene delivery strategies.

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